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STUDIES ON THE SAPONIN OF SOY-BEAN. PART I.

By

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(Received July 15th., 1929).

Isolation of the Saponin.

The air dried soy-bean are powdered in a mill, extracted with ether, dried and boiled in the water bath with 80% alcohol (7 l. per 2kg.) for 2 hours under the reflux condenser. After repeating this treatment 3 times, each filtrate is evaporated under the reduced pressure. The precipitate, produced by the addition of HCl (1 : 1) to the evaporated residue, is dissolved in 2% KOH, filtered, again precipitated by HCl and washed by decantation several times with dil. HCl. The washed precipitate is dried in the vacuum desiccator over the sulphuric acid and extracted with ether and then peteroleum ether each twice times. The residue is dissolved in 80% alcohol and purified using animal charcoal. Thus the crude saponin is obtained (yield 0.114%). After recrystallization from 80% alcohol or acetone, the saponin forms squama, melting at 222—4° (incorr.).

Molecular Formula and Property of the Saponin.

Analysis :

	Subst (g.)	CO_2(g.)	H_2O(g.)	%C	%H	%O
1.	0.1432	0.3108	0.1008	59.19	7.82	
2.	0.1264	0.2740	0.0892	59.12	7.84	
3.	0.1510	0.3268	0.1012	59.03	7.45	
4.	0.1514	0.3268	0.1068	58.86	7.84	
5.	0.1554	0.3370	0.1060	59.14	7.58	
6.	0.1598	0.3442	0.1104	58.78	7.68	
aver.				59.02	7.70	33.28

Molecular weight :

	Subst.(g.)	N/10 NaOH (c.c.)	N/10 KOH (c.c.)	Mol. weight.
1.	0.2136	2.2088		967
2.	0.1510	1.5562		970
3.	0.1730		1.7892	967
4.	0.2096		2.1862	959

Molecular formula :

From the results above described, the molecular formula is given as $C_{49-52}H_{70-82}O_{21}$.

Specific rotation :

0.1658 g. of saponin is dissolved in 25 c.c. of absolute alcohol and observed by sodiumlight. $l=1$, $\alpha=0$.
$l=2$, $\alpha=0$.

Hemolysis :

	Saponin										Control				
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5
c.c. of saponin Sol.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0	0	0	0	0
c.c. of 0.9% NaCl Sol.	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.0	1.0	1.0	1.0
c.c. of corpuscle Sus.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Result 1.00%	##	##	##	##	##	##	##	#	#	+	−	−	−	−	−
Result 0.10%	−	−	−	−	−	−	−	−	−	−					
Result 0.01%	−	−	−	−	−	−	−	−	−	−					

Colour reaction :

1. By conc. H_2SO_4 ; brown→red→reddish violet.
2. By acetic acid anhydride and conc. H_2SO_4 ; violet.

The Products by the Hydrolysis of Saponin.

The hydrolysis of this saponin is very difficult. If we use the solution of 80% alcohol–5% sulphuric acid, it requires 100 hours for the complete hydrolysis and if we raise the concentration of sulphuric acid to 10%, it requires yet 40 hours.

(1) *Sapogenins.*

(a) Sapogenin A.

The saponin is hydrolyzed for 100 hours by the method above described. After evaporation of alcohol, the crude sapogenin is precipitated by adding water, washed to remove monosaccharides and recrystallized from boiling absolute alcohol. Thus sapogenin A, m. p. 240∼1° (incorr.), is obtained. Yield : 10%. The reaction of Liebermann is positive.

Sapogenin A is considered as the codensation product of sapogenin B, having the molecular formula, $C_{64-70}H_{94-110}O_5$.

	Subst.(g.)	H_2O(g.)	CO_2(g.)	%H	%C
1.	0.1554	0.1476	0.4632	10.55	81.29
2.	0.1472	0.1444	0.4382	10.90	81.19
3.	0.1620	0.1556	0.4812	10.67	81.01
aver.				10.71	8 1.16

(b) Sapogenin B.

The crude sapogenin obtained by the same treatment as sapogenin A, is recrystallized from boiling absol. alcohol and sapogenin B is obtained crystallizing in long prisms (not same as the sapogenin A). Sapogenin B melts at

234~6° (incorr.), gives the reaction of Liebermann and contains yet the crystal water at 100° in a vacuum tube but loses it completely at 142° in a vacuum tube.

Analysis :

	Subst.(g.)	H_2O(g.)	CO_2(g.)	%H	%C
1.	0.1436	0.1360	0.4208	10.52	79.92
2.	0.1451	0.1391	0.4260	10.65	80.07
3.	0.1278	0.1252	0.3750	10.88	80.03
aver.				10.68	80.01

Molecular weight :

Subst.(g.)	Camphor(g.)	△T°	Mol. weight.
0.0154	0.1486	8	518
0.0144	0.1508	8	478

Molecular formula :

From the above results, the molecular formula is given as $C_{32-33}H_{48-56}O_3$.

Treatment by alcoholic potash :

0.2450 g. of sapogenin B is dissolved in the mixture of 95% alcohol and N/2 KOH, boiled for 8 hours in the water bath under the reflux condenser and after cooling titrated with N/5 H_2SO_4 using phenolphthalein as indicator. But there is no consumption of alkali.

Catalytic reduction :

0.7780 g. of sapogenin B is dissolved in 40 c.c. of glacial acetic acid and reduced using palladiumchloride as catalyser. But there is no absorption of hydrogen.

Benzoylderivative :

0.5 g. of sapogenin B is dissolved in pyridine and added with the theoretical quantity of benzoylchloride drop by drop cooling with ice water. After 15 hours, the benzoylderivate is precipitated by adding excess of ether. Yield : 0.5 g. It is a very hygroscopic crystal melting at 232~4°.

Acetylderivate :

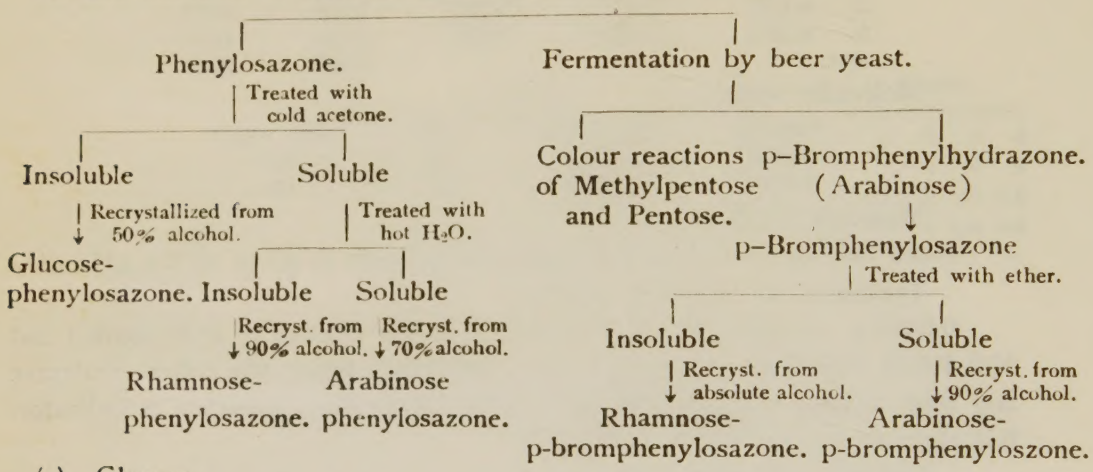
1 g. of sapogenin B is dissolved in the mixture of acetic acid anhydride (10 g.) and dehydrated sodiumacetate (1 g.) and boiled for 3 hours in the oil bath under the reflux condenser. The reaction mixture is poured into 50 c.c. of water and boiled. After cooling, the white precipitate is filtered and recrystallized from boiling alcohol or acetone. It is a crystal powder melting at 132~7°. The result of analysis shows to be triacetylderivative.

Subst. 0.1362 g. H_2O 0.1136 g. CO_2 0.3646 g.		%H=9.52	%C=74.95
Cal. for	$C_{33}H_{53}O_3(CH_3CO)$	10.37	77.77
	$C_{33}H_{52}O_3(CH_3CO)_2$	9.97	76.28
	$C_{33}H_{51}O_3(CH_3CO)_3$	9.61	75.00

(2) *Monosaccharides.*

Aftear the hydrolysis of saponin, the reaction mixture is evaporated under the reduced pressure, added with water and filtered to remove the sapogenin. The filtrate is neutralized with bariumcarbonate, bariumhydroxide or calcium-

carbonate (only the case of the fermentation of hexose) and filtered off to separate Ba-sulphate or Ca-sulphate. The filtrate is evapolated to syrup and extracted with 80% alcohol. The alcoholic solution is evaporated to dryness under the reduced pressure and then extracted with 95% alcohol, next absol. alcohol. The alcoholic solution is evaporated and examined as follows. (The Ba-salt insoluble in alcohol will be reported another day.)



(a) Glucose.

1. Fermentation by beer yeast.
2. Seliwanoff's reaction is negative (not ketose).
3. No phenylhydrazone (not mannose).
4. Phenylosazone melts at 200~2° (not galactose).

(b) Rhamnose.

1. Rosenthaler's reaction is positive.
2. Akabori's reaction is positive.
3. Phenylosazone melts at 176~8°.
4. p-Bromphenylosazone melts at 213~5°.

(c) Arabinose.

1. Bial's reaction is positive.
2. Akabori's reaction is positive.
3. Phenylosazone melts at 164~6°.
4. p-Bromphenylhydrazone melts at 164~5°.
5. p-Bromphenylosazone melts at 182~3°.

Estimation of the Hydrolyzed Products.

(1) *Sapogenin.*

The saponin is hydrolyzed completely by the method above described and the reaction mixture is evaporated to expel the alcohol. The sapogenin, precipitated by the addition of water, is filtered on the Gouch's crucible, dried

at 120° and weighed.

	Saponin(g.)	Sapogenin(g.)	Sapogenin(%)
1.	0.7612	0.4434	58.25
2.	0.8074	0.4598	56.95
3.	0.7310	0.4106	56.17
4.	0.7550	0.4182	55.38
aver.			56.68

(2) *Glucose.*

4.4218 g. of saponin hydrolyzed with 150c.c. of 80% alcohol–5% sulphuric acid for 100 hours. The sapogenin and Ca–sulphate, precipitated by treatment with Ca–carbonate, are filtered off and washed completely by hot water. Each filtrate is mixed, evaporated and filled up to 50 c.c.. And a certain quantity of this solution was fermented at 30° by the beer yeast using Lohnstein's apparatus and gravimetric fermentation apparatus. The found % of glucose is 12.72 from the former and 11.58 from the latter. But during the hydrolysis, 10% of glucose is decomposed. ∴ % glucose=13.99.

(3) *Rhamnose and Arabinose.*

These 2 monosaccharides are determined by th methode of Krüger–Tollen.

Saponin (g.)	Furfurol–phloroglucide	Methylfurfurol–phloroglucide	Arabinose (%)	Rhamnose (%)
4.4218	0.3476	0.2096	8.92	7.34
0.5808	0.0250	0.0532	5.82	16.13

Discussion.

From the results above described, it is a established fact that the saponin of soy-bean consists of each one molecule of sapogenin, glucose, rhamnose and arabinose. But although the number of carbon is taken just the same, the numbers of hydrogen and oxygen are not the same, for example.

$$\underset{\text{Saponin}}{C_{50}H_{72-78}O_{21}} + 3H_2O = C_{50}H_{78-86}O_{24}.$$

$$\underset{\text{sapogenin}}{C_{35}H_{50-52}O_3} + \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{rhamnose}}{C_6H_{12}O_5} + \underset{\text{arabinose}}{C_5H_{10}O_5} = C_{50}H_{84-86}O_{19}.$$

This inconsistency appears to issue from the dehydration of sapogenin B for the long hydrolysis, but from the result of catalytic reduction of sapogenin B it is acertained that there is no double bond in the side chain. Moreover the acidity of saponin disappears by the hydrolysis, because the oxygens of sapogenin all form the hydroxyl groups. Therefore it is right and proper to consider that an organic acid is separated by the hydrolysis. But the destillate of the steam distillation of hydrolyzed mixture does not give acid reaction against litmus paper. So it is acertained that this acidic substance is not volatile acid but non–volatile acid described above as the Ba–salt insoluble in alcohol. If I take mesoxalic acid as this organic acid for example, the equation of

hydrolysis is as follows.

$$C_{52}H_{80}O_{21} + 4H_2O = C_{32}H_{50}O_3 + C_6H_{12}O_6 + C_6H_{12}O_5 + C_5H_{10}O_5 + C_3H_4O_6 = C_{52}H_{88}O_{25}$$

saponin — sapogenin — glucose — rhamnose — arabinose — mesoxalic acid

I promise here there will be another chance to report on this acid.

It is a pleasure to express my gratitude to Prof. T. Yabuta, for his continued advice and encouragement.

INVESTIGATION ON THE BIILMANN'S QUINHYDRONE ELECTRODE. IV.

COMPARATIVE STUDY OF QUINHYDRONE AND HYDRO-QUINHYDRONE ELECTRODE.

By

ARAO ITANO and SATIYO ARAKAWA

(Received July 21st., 1929.)

The practical value of Biilmann's quinhydrone and hydro-quinhydrone electrode in various solutions together with the test on the simplified, saturated KCl-calomel electrode in comparison with the standard N/10 KCl-calomel electrode were investigated.

The investigation was undertaken because it was pointed out recently by some investigators, Biilmann, Grossman, Shikata and others that the quinhydrone electrode in some instances is less reliable than the hydro-quinhydrone electrode especially in presence of some such substances as glucose, alcohol ammonia and others.

The simplified, saturated KCl-calomel electrode was tested against the standard N/10 KCl-calomel electrode using three different kinds of buffer solutions.

The following summary and conclusions were reached:

1. Under all the conditions tested, the quinhydrone electrode seems to give the accurate results providing that the readings are taken soon after the quinhydrone is added viz. within one minute.

2. On the average, the lower pH values were obtained by the hydro-quinhydrone electrode especially in those solutions of which pH values > 7.

3. The quinhydrone electrode is prefered to the hydro-quinhydrone since

the manipulation is simpler.

4. The simplified, saturated calomel electrode gives slightly larger pH in all cases viz. pH 0.06 in with the H_2 and quinhydrone electrodes and pH 0.04 with the hydro-quinhydrone respectively. The mean error is so small viz. ± 0.0016 to 0.0025 that the simplified, saturated calomel electrode may be used satisfactorily for the most of biological investigations.

STUDIES ON BACILLUS THERMOFBRINCOLUS N. SP.

I. DESCRIPTION OF THE ORGANISM.

By

Arao Itano and Satiyo Arakawa.

(Received July 24th, 1929).

Morphological and cultural description of a new thermophilic, cellulose fermenting bacteria, Bacillus thermofibrincolus n. sp., is given together with a review on some of the similar organisms.

A comparative study of some of the thermophilic cellulose fermenting bacteria which are closely related is given as Table I:

Table I.

(Comparative Study of Some Thermophilic Cellulose Fermenting Bacteria)

Authors.	Langwell & Lymn	Itano & Arakawa	Viljoen, Fred & Peterson.
Name of organism.	——	Bacillus thermofibrincolus.	Closteridium thermocellum.
Size of rods, μ.	0.4 × 0.4	4.2 × 0.5	5.0 × 0.4
Size of spore, μ.	——	1.5 × 0.9	0.9 × 0.6
Flagella.	none.	peritriche.	peritriche.
Gram stain.	−	+	−
Nutrient broth.	grow.	grow.	grow.
Glucose broth.	gas.	no gas, acid, light membrane, abundant viscid sediment.	gas and acid, pellicle, sediment.
Nutrient agar.	glistening, moist, butyrous.	moist translucent. butyrous.	moist, glistening, butyrous.

Agar colony.	surface & bottom small.	ditto.	ditto (starch agar)
Potato.	vellow, moist, potato browned.	gray, white, moist, potato no change.	yellow, poteto no change.
Milk.	acid, coagulation, in 5 days, no digestion, reduce litmus.	acid-coagulation in 3 days, wheyed, gas, reduce litmus.	slight acid coagulation in 3 days, wheyed, gas.
Indol.	——	positive.	negative.
Catalase.	——	positive.	negative.
Methyl acetyl carbinol.	——	negative.	negative.
Best N source.	——	albumin.*	peptone.
Fermentation of Carbohydrates.	starch, hemicellulose, glucose, xylose.	starch, hemicellulose, raffinose, glucose, lactose. maltose, mannose, galactose, fructose, sucrose, xylose, arabinose, salicine.	ditto.

* The action on the albumin will be reported in the next paper.

The photographs, in original paper, showing the vegetative cells, sporangia, flagella and fermentation of the filter paper in the test-tubes are accompanied.

STUDIES ON OYSTERS (I).

CONSTITUENTS OF OYSTERS.

By

Wataru. SHIMIDZU.

(Received July, 28th., 1929.)

These chemical studies on the two kinds of Japanese oysters, Ostrea gigas Thumb. & Ostrea denselamellosa Lisch. were inangurated in May 1926, extending over two years. The results thus produced are as follows:

1. As for the ratio of the amount of flesh to shell, the Ostrea gigas has a higher value, though relatively, than the Ostrea denselamellosa. The flesh of the latter is wasted especially in summer.

2. The analytic results of the flesh show that the O. denselamellosa contains much more nitrogen than O. gigas, but on the contrary, with regard to the quantities of the other elements—ash, soluble matters in ether and alcohol, glycogen—, the former is inferior to the latter.

The O. gigas, which lives in less salty water, contains very large quantity of ash ; this fact seems to indicate that it has large amonnt of mineral substances with the rapid growth of the shell.

These oysters contain much more glycogen than the other marine shells ; but this glycogen, of course, is not always constant in the different seasons when they are collected, and also it will be properly supposed that it is changeable every moment when they are being stored, because it is a storing substance like fat or oil.

3. The ash of oysters was analysed and the following results have been obtained.

	In 100 g. of ash.	
	Ostrea gigas.	Ostrea denselamellosa.
Carbon	0.593	0.0
Sand & SiO_2	5.897	4.530
SO_3	3.827	7.042
K_2O	23.148	16.281
Na_2O	16.799	18.914
Fe_2O_3	3.320	2.043
CaO	4.495	5.783
P_2O_5	30.502	21.564
MgO	4.945	4.534
Mn_2O_3	0.134	0.190
Cl	1.769	15.248

These results prove that the chlorine-content in the ash of the O. gigas is very deficient, but the phosphoric acid content is remarkably rich. From the above, most of the phosphoric acid in the O. gigas may be said to have taken the chlorine's place for an anion. The potash is one of the predominant constituents in the ash, and exists, it seems, mostly in the form of the phosphate in the oysters.

4. The nitrogen of the extract of the flesh with ether and alcohol was respectively measured and the various forms of nitrogen in the residue were also determined : —

	In 100 g. of the anhydrous oysters.	
	Ostrea gigas.	Ostrea denselamellosa
Total nitrogen.	7.135	9.600
Nitrogen soluble in ether.	0.127	0.445
Nitrogen soluble in alcohol.	1.601	1.692
Nitrogen not dissolved in conc. HCl.	0.257	0.217
Nitrogen dissolved in conc. HCl.	5.220	7.350
Amide nitrogen.	0.409	0.677
Humin nitrogen.	0.148	0.150
Mono-amino nitrogen.	3.481	4.977
Di-amino nitrogen.	1.427	2.060
Arginin nitrogen.	0.567	0.825

Cystin nitrogen.	0.339	0.460
Histidin nitrogen.	0.440	0.619
Lysin nitrogen.	0.081	0.158

From these results, it will be concluded that the flesh of the oysters has a high nutritive value, as it contains Histidin, Cystin, Lysin in abundance.

STUDIES ON OYSTERS (II).

THE OPTIMUM HYDROGEN ION CONCENTRATION AND THE OPTIMUM TEMPERATURE OF THE GLYCOGENASE OF THE OYSTER.

By

Wataru. SHIMIDZU.

(Received Sept, 6th , 1929.)

The powerful, yet unstable, glycogenase were isolated from the flesh of Ostrea gigas, and the various characters on its activity were investigated :

1). The optimum hydrogen ion concentration for the glycogenase action of the oyster in acetic-soda buffer was found to be at or near pH 5.0, and in citrate buffer it was at pH 5.5. These experiments were carried out under the identical conditions, viz. the enzyme was allowed to act upon glycogen solutions at 30°C for 48 hours, and at the end of the time their reducing powers were determined. So the above results prove that the optimum hydrogen ion concentration is varied with the buffer solution used.

But, on the other hand, when phosphate buffer was used, the optimum hydrogen ion concentration was shown to be at least as high as that indicated by pH 6.5~6.8 at the first hour ; but its maximum point being moved little by little with time, it was shown to be at pH 5.5 after 24 hours, and lower than it after 48 hours.

2). And its reaction velocity was turned down with time. From these results, we can suggest these two facts.

The first—, if we grant that the enzyme is active but easily destroyed at or near pH 6.8 ; and at or near pH 5.0, it is less active and it is dulable ; thus, as a necessary consequence, we can understand that the diastatic products are greater at the higher hydrogen ion concentration in the long run.

The second—, it is quite possible that there are really two or more amylases

present in this preparation. The one which optimum hydrogen ion concentration is at or near pH 6.8, has a strong diastatic power, but it is unstable; the other which optimum hydrogen ion concentration is at pH 5.0～5.5, has a weaker diastatic power, but this is more stable. If we admit that the former is active during the first hour, but is destroyed by warming much more rapidly than the latter; these facts that the maximum diastatic power is altered with time and shown to be at pH 5.5 or less after a long time, would be simply explained.

3). The optimum temperature for the glycogenase of Ostrea gigas was determined to be at 45°C, in acetic-soda buffer (pH 5.5), after 1 hour; but its optimum was altered gradually with time, and indicated to be at or near 40°C, after 24 hours.

And the reaction velocity is shown to be in accordance with the monomolecular equation in a few hours. However, the velocity is decreased with time; and the higher the temperature is, the lower the enzyme action becomes.

STUDIES ON OYSTERS (III).

THE INFLUENCE OF SALTS ON THE GLYCOGENASE.

By

Wataru. SHIMIDZU.

(Received Sept., 20th., 1929.)

The glycogenase of the flesh of Ostrea gigas increases its activity in the presence of the small quantities of sodium chloride, potassium chloride and calcium chloride; and the optimum is apparently about 0.1%. By the addition of the salts, no alteration of the pH-value was fouud in the solution. It will be seen that this amylase resembles closely the amylases of the other animal origin in being extremely sensitive to the presence of these salts.

The effect of potassium chloride is shown to be similar to that of sodium chloride; and the effects of calcium chloride and sodium chloride containing a trace of calcium chloride, are found to be more sensitive than the formers.

When Ringer's solution is used instead of these salt solutions, the enzyme action is shown to be accelerated at higher degrees. And even in the presence

of such a concentrated solution as 1 or 2% (this concentration is to be compared with that of the sea water and the body liquid), its activity not only to be less depressed, but on the contrary, after a long time, its activity in creases enormously.

This result differs from these obtained with the other salts. When the mixture of various salts such as Ringer's solution, is added to the solution, it seems that there must be taken place the antagonistic action between those salts. All living things are generally injured by presence of single salt, but when there are two or more salts it is not injured. It is, therefore, suggested from these facts that one of the mechanisms of antagonistic action of salts at the biological world may be explained by the action of enzymes.

THE FORMATION OF KOJIC ACID BY ASPERGILLUS ORYZAE.

PART I. THE FORMATION OF KOJIC ACID FROM PENTOSES, SUGAR ALCOHOLS AND GLUCONIC ACID.

By

Hideo Katagiri and Kakuo Kitahara.

(Received Sept., 15th, 1929)

It was first noted by Dr. Saito (1907) that Aspergillus Oryzae grown on steamed rice produced a special acid. This acid was named kojic acid by Dr. Yabuta (1912) and was determined by him (1916, 2 and 1924) to be a γ pyrone derivative (m. p. 151～152°); $C_6H_6O_4$ (5–hydroxy–2–hydroxymethyl–γ–pyrone) and was found that its diacetyl derivative melted at 101～102°.

Many authors have already discussed the question whether the formation of kojic acid reveals any remarkable difference between the various moulds or sugars. Dr. Saito (1907) observed the formation of the special acid by Asp. oryzae with cane sugar, maltose, glucose, galactose or glycerol but not with mannitol. Dr. Yabuta (1916, 1) pointed out that not only Asp. oryzae but also Asp. candidus, Asp. nidulans and Asp. Wenti produced a large amount (12.7～20.5% of theoretical value) of kojic acid from glucose or maltose, but a considerably small amount of the acid was obtained when the moulds grew on fructose solution. However, no formation of kojic acid from these sugars

was observed by Asp. niger, Asp. glaucus, etc.

On the other hand, Traetta-Mosca (1914) observed that Asp. glaucus grown on cane sugar or invert sugar solution produced an acid having a formula of $C_6H_6O_4$ which he suggested to be γ lactone of trihydroxyhexadienolic acid. Traetta-Mosca and Preti (1921) subsequently noted that Asp. glaucus formed this acid from glycerol. Recently Wijkman (1924) showed that this acid (m. p. 154°) was probably 2–hydroxymaltol, since hydroglucal was obtained by hydrogenation of the acid.

In the present paper, experiments were instituted with glycerol, sugars, sugar alchohls, sugar acids and many other substances, in order to ascertain from which substance kojic acid could be formed by Asp. oryzae, and whether kojic acid would differ from the acid described by Traetta–Mosca.

Methods and Results.

In order to ascertain whether kojic acid would be formed from a given substance by Asp. oryzae (Higuchi blue), it is necessary in the first place to decide on an appropriate standard method of culturing the mould. The amount of kojic acid produced by the mould is not the same when various concentrations of $(NH_4)_2SO_4$ or sugar are employed. The pH value of the medium also exerts a considerable effect upon the formation of kojic acid.

Effect of the Concentrations of $(NH_4)_2SO_4$ *or Carbon Source.*

Simple examples are given below with the case of glycerol, in which various concentrations of glycerol or $(NH_4)_2SO_4$ are employed while the other mineral matters (0.1% KH_2PO_4, 0.01% $MgSO_4$ and 0.01% $CaCl_2$) are kept constant.

$(NH_4)_2SO_4$ %	Glycerol %	Kojic acid	
		after 8 days	after 10 days
0.05	1	0	0
0.05	5	+	0.014%
0.25	5	0	0

It will be seen that the formation of kojic acid is observed only with the medium containing a least amount of $(NH_4)_2SO_4$ and a greater amount of glycerol. These relations are well brought out in the following experiments.

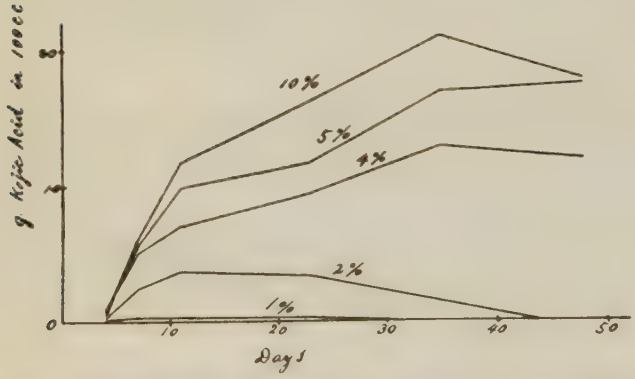
The relation between the amount of kojic acid formed and the concentration of $(NH_4)_2SO_4$ is very clearly seen with the cases of 100 c.c. of 5% lactose media, as is illustrated in Table I, that the formation of the acid increases as the concentration of $(NH_4)_2SO_4$ diminishes, while the weight of the mould decreasing considerably as does the concentration of $(NH_4)_2SO_4$.

Table I.

$(NH_4)_2SO_4$ %	g. Kojic acid in 100 c.c. after						Weight of Mould after 30 day's Incubation
	4	6	8	14	18	30 days	g.
0	0	+	+	0.007	0.008	0.016	0.045
0.01	0	0	+	0.017	0.028	0.050	0.148
0.02	0	0	0	0	0	0	0.206
0.05	0	0	0	0	0	0	0.213

When the concentration of $(NH_4)_2SO_4$ is kept constant, it will be seen in Fig. I that the amount of kojic acid formed depends on the concentration of glucose.

Fig. I



With 1% glucose, the formation of kojic acid is very trace. A considerable amount of kojic acid is formed with 2% glucose at about 10 day's incubation, but after this period the acid decreases fairly rapidly until about 40 days when no kojic acid is observed, as was pointed out by Dr. Yabuta (1916, 1) that kojic acid was assimilated by Asp. oryzae. With 4–10% glucose, the formation of kojic acid increasing at first (during 10 days' incubation) rapidly, and then gradually. Among the various concentrations of glucose, the maximum formation of kojic acid is observed with 5% solution, from which the yield in 48 days attains to 34% of glucose employed, i.e. 43.18% of theoretical value.

Effect of pH *Value.*

Table II in which N/10 phosphate is employed for the buffer to 5% glucose solution containing 0.05% $(NH_4)_2SO_4$ (the other mineral matters are the same as mentioned above) shows that the optimum pH value for the formation of kojic acid about 2.4, while the optimum for the growth of the mould is about 5.0.

Table II.

pH Value			g. Kojic acid in 100 c.c. after				Growth of Mould after	
Beginning	after 13 days	after 16 days	4	8	13	16 days	2 days	16 days (g.)
1.7	1.7	1.7	0	0	0.02	0.030	scanty	0.058
2.7	2.4	2.2	0	0	0.42	0.500	moderate	0 202

4.5	3.0	3.0	0	trace?	0	0.003	abundant	0.210
6.3	3.7	3.7	0	0	0	0.002	abundant	0.205
7.3	6.4	6.0	0	0	0	+	abundant	0.168
10.0	7.5	7.2	0	0	0	0	scanty	0.068

In the present paper, experiments were always carried out with a liquid medium of the following composition: 5.0 g. of a given substance, 0.05 g. $(NH_4)_2SO_4$, 0.1 g. KH_2PO_4, 0.01 g. $MgSO_4$ and 0.01 g. $CaCl_2$ were dissolved in water up to 100 c.c., inoculated with spores of Asp. oryzae and incubated at 29～31°. Any special buffer solution had not been used, but the initial pH value of the liquid was in each case about 4.5 and the final pH value was about 2.2 whenever a considerable growth of the mould has been observed. This range of pH values probably approaches to the optimum zone for the formation of kojic acid.

In the early period of incubation, the amount of kojic acid was calorimetrically determined; the red colour produced by the addition of $FeCl_3$ solution to a certain amount of the liquid culture, was compared with standard kojic acid solutions which are made from the acid (m. p. 150～151°) carefully prepared with koji-extract. After a certain day's incubation, the whole liquid was filtered and acidified with H_2SO_4, extracted with ether for 2～3 days. The crude kojic acid obtained from the ether solution, was washed with petroleum ether, dissolved in water and again extracted with ether. Needle crystals having m. p. and mixed m. p. 150～151° were, in each case shown in Table III, obtained from the final ether solution.

For a further identification, the kojic acid obtained from xylose, sorbitol, gluconic acid or from glycerol was converted into diacetyl derivative by boiling for 2 hours with acetic anhydride. The diacetyl derivative (needle crystals) after crystallisation from alchohl, melted, in each case, alone or admixture with the specimen derived from the standard kojic acid at 101°.

Table III.

The amount of kojic acid obtained from the ether extraction of 100 c.c. of 20 days' liquid culture containing 5 g. of a given substance.

Substance	Kojic acid	
	g.	Yied (%)
Maltose	1.70	40.8
Cane sugar	1.45	34.8
Glucose.	0.75	18.1
Inulin	0.65	14.8
Fructose	0 40	10.2
*Sorbitol	0.40	10.2
*Dulcitol	0.40	10.2
Xylose	0.40	10.2
△Gluconic acid	0.34	9.4

Mannose	0.23	5.8
Glycerol	0.16	4.2
Arabinose	0.08	2.0
Galactose	0.04	1.0
*Inositol	0.04	0.9
Na-glycero-β-phosphate	+	−

* and △ represent 14 and 40 days' incubations respectively.

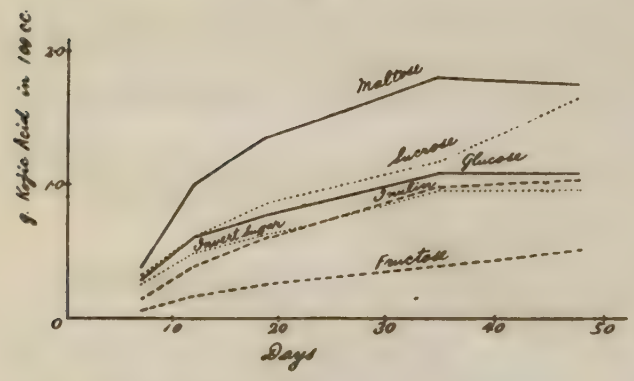
Carbohydrates.

(a) Di-and polysaccharides: It will be seen in Table III that with the disaccharides such as maltose or cane sugar the maximum formtion of kojic acid is observed among all the substances employed. However, a considerably small amount of kojic acid is obtained with inulin, and no formation is observed with lactose except the case, as is shown in Table I, in which very slight amount of $(NH_4)_2SO_4$ is used.

(b) Hexoses and Pentoses: Not only with the disaccharides but also with the monosaccharides, it will be seen in Table III that the amount of kojic acid formed from sugars depends on their configurations; with glucose the yield is about 20 times of that with galactose and with xylose it is 5 times of arabinose, while the growth of the mould in all cases does not differ greatly from one another.

It is interesting to note as is shown in Fig II that with di- or polysccharides the amount of kojic acid formed is always greater than that with their hydrolysates; a considerable difference in the formation of kojic acid is pointed out between 4.75 g. of maltose and 5 g. of glucose, 4.5 g. of inulin and 5g. of fructose or 4.75 g. of sucrose and 5 g. of invert sugar in 100 c.c.

Fig. II



(c) Methyl glucoside and methyl pentose: With α-methyl glucoside or with rhamnose, the growth of the mould is not so well as that with glucose, and any kojic acid is not obtained from these substances, even when for the nitrogen source, various concentrations, e. g. 0, 0.002, 0.01 and 0.05g. of $(NH_4)_2SO_4$ in 100 c.c. are chosen.

(d) Trioses: While a considerable amount of kojic acid is obtained from glycerol as is shown in Table III, no direct evidence for the formation

of the acid is observed with glyceraldehyde prepared by the method described by Fenton and Jackson (1899) or with dihydroxyacetone prepared by Fischer and Tabel's method (1887), when these substances are employed as 0.8% solution in the presence or absence of $(NH_4)_2SO_4$ and a well grown mould on lactose is inoculated to these solutions.

Polyhydric alcohols.

(a) Hexahydric alcohols: With sorbitol or with dulctitol a considerable amount of kojic acid is formed as is shown in Tabel III, but with mannitol, as was already noted by Dr. Saito (1907), no formation of kojic acid is observed. And the growth of the mould on mannitol is not so well as that on the other alcohols or on sugars.

It is found that not only with sugar alcohols such as sorbitol etc., but also with hydro-aromatic compound such as inositol, a noticeable amount of kojic acid is formed as is shown in Table III.

(b) Tetra-and dihydric alcohols: No formation of kojic acid is observed with erythritol or with ethylene glycol although the growth of the mould with these alcohols does not differ greatly from that with sorbitol etc.

(c) Glycerol and glycerophosphate: There is no evidence of the formation of the acid described by Traetta-Mosca (1914) and Wijkman (1924), since the acid obtained from glycerol melts alone or in admixture with kojic acid carefully prepared from koji-extract, at 150~151° and the diacetyl derivative has m. p. and mixed m. p. 101°, as was already mentioned.

A trace of kojic acid is obtained with Na-glycero-β-phosphate in the presence or absence of KH_2PO_4, and a noticeable amount of phosphate is observed to be produced from the glycerophosphate by the mould. Therefore, in the first place glycerol is probably formed by hydrolysis of the glycerophosphate and then the formation of kojic acid is taken place from the glycerol, but the exact changes involved in the formation of kojic acid are under investigation.

Hydroxy acids.

(a) Gluconic and saccharic acids: It will be seen in Table III that with 5% gluconic acid, the amount of kojic acid formed is about the half of that with 5% glucose. Even with 2% of these substances, nearly the same ratio of the yields is observed as is illustrated below.

Substance	g. kojic acid in 100 c.c. after					
	5	7	10	15	35	48 days
2% glucose	0.106	0.24	0.35	0.33	0.14	0
2% gluconic acid	—	+	0.02	0.10	0	0

No formation of kojic acid is observed with saccharic acid or with its

sodium hydrogen salt.

(b) Dihydroxy acids: With glyceric acid prepared from glycerol in the way described by Mulder (1876) or with its calcium salt, no formation of kojic acid is observed, while a considerable amount of kojic acid is obtained from glycerol as is mentioned above.

No formation of the acid is again observed with sodium tartrate.

(c) Monohydroxy acids: Any kojic acid is not obtained with the following substances, i. e. Na–glycollate, Na–lactate or Na–tartronate, although the growth of the mould, in each case, is moderate.

Fatty acids and Ketonic acids.

With Na–acetate, Na–propionate, oxalic acid, Na–malonnate, Na–pyruvate or with ethylacetoacetate, no formotion of kojic acid is in every case observed.

Aldehydes, Ketones, Alcohols and Ketonic Alcohols.

No formation of kojic acid is again observed with acetaldehyde, acetone, diethyl–acetal, propyl alcohol, iso–propyl alcohol, allyl alcohol or acetol prepared from chloracetone by Nef's method (1904). With actaldehyde, not only in absence of any other carbon source, but also in the presence of lactose, no formation of kojic acid is observed as is shown in the following experiments in which a well grown mould on glucose for 3 days is employed to each medium.

Substance	g. Kojic acid in 100 c.c. after			
	1	3	5	15 days
5% lactose + few drops of 5% acetaldehyde (added every days)	0	0	0	0
5% lactose	0	0.005	0.04	0.30
water	+	0	0	0

Compounds containing Amino–Group.

With none of the following substances; urea, thiourea, glycocoll, alanine, aspartic acid, asparagine or ethylene diamine, the formation of kojic acid is observed.

Effect of $CaCO_3$ *or* $CaSO_3$.

The chemical change involved in the formation of kojic acid is under investigation, but it is worth to note that the amount of kojic acid formed from a given substance is greatly decreased when $CaCO_3$ is added to the medium as is shown, for an example, in the following experiments.

Substance	g. kojic acid in 100 c c. in 20 days.
5% glucose	0.64

5% glucose + $CaCO_3$	0.042
5% gluconic acid	0.34
5% gluconic acid + $CaCO_3$	0.003

It will be seen that $CaCO_3$ exerts a similar effect both on glucose and on gluconic acid ; therefore it is probably suggested that gluconic acid is an intermediate substance for the formation of kojic acid from glucose, but it is rather probable to suggest that the effect of $CaCO_3$ in diminishing the formation of kojic acid is attributable to the chage in pH value of the medium, as was already shown in Table II.

Any direct evidence does not afford to the formation of kojic acid from acetaldehyde in the presence or absence of any other carbon source, as was already discussed. However, acetaldehyde is detected in the various culture media containing 2% Na_2SO_3, as is illustrated below, when well grown mould on glucose is inoculated to the media.

Table IV.

Substance	Acetaldehyde							g kojic acid in 100 c c. (20 days)
	2	4	6	8	10	15	20 days	
Glucose	+	++	++	+++	+++	++	+	0.28
Fructose	+	++	++	+++	+++	++	+	0.11
Galactose	+	+	++	++	++	++	+	0.04
Lactose	0	+	+	+	+	+	0	trace
Arabinose	0	?	?	0	0	0	0	0.08
Xylose	0	0	0	0	0	0	0	0.14
Glycerol	0	+	++	++	++	+++	++	0.23
Pyruvic acid	0	0	0	0	+	0	0	0

It will be seen in Table IV that with the hexoses and with glycerol the amount of aldehyde formed is so considerable that the maxium yield attains to 1.7% of sugar, while with lactose only a slight amount of acetaldehyde is detected, and no aldehyde is observed with pentoses. Any simple relation is not found to exist between the amount of aldehyde and that of kojic acid, but it is pointed out that the formation of kojic acid diminishes in general in the presence of Na_2SO_3, when the results in Table IV are compared with those in Table III, e. g. with the cases of glucose, fructose and xylose the yield is in each case reduced to about one third.

With galactose or arabinose, no difference is found in the formation of kojic acid between in the presence and absence of Na_2SO_3, and with glycerol more kojic acid is formed in the presence of Na_2SO_3. However, in discussing the amount of kojic acid formed with these substances from which fairly slight amount of the acid is obtained in the absence of Na_2SO_3, a certain correction for the amount produced from the former culture medium (glucose) must be taken into account, since in the present experiments a well grown mould on

glucose is chosen.

In order to eliminate the amount of kojic acid due to the history of the mould, as is illustrated in Table V, further experiment in which Na_2SO_3 was added to 5% glycerol culture after several days' incubation when the mould had grown well, was made.

Table V.

Substance added to 5% glycerol	g. Kojic acid in 100 c c. (days after the addition of substance)			pH (20 days)
	0	10	20	
Na_2SO_3	0.025	0.042	0.11	6.85
water	0.018	0.20	0.32	2.60

It will be seen in Table V that the formation of kojic acid is considerably reduced by the addition of Na_2SO_3, as was observed in the cases of glucose etc. The diminishing effect of Na_2SO_3, in part probably suggests that acetaldehyde is an important substance in the formation of kojic acid, but it appears that the effect is attributable, in a great measure, to the change in pH value of the medium.

Further experiments are carried out with glucose and glycerol in order to ascertain whether dimedone exerts any special effect on the formation of kojic acid when it is added up to 0.4% to the culture media. It is found that not only the formation of kojic acid but also the growth of the mould is reduced, in both cultures, in the presence of dimedone, although the diminishing effect is not the same with the cultures; in the case of glucose culture the effect is not so great as with the case of glycerol in which the formation of kojic acid in 30 days is reduced from 0.44 g. to 0.056 g. in 100 c.c. and the growth of the mould is also greatly affected. Neither acetaldehyde nor any other aldehydes are not in both cases fixed by the addition of dimedone.

It is worth to note that the formation of kojic acid is not observed with Na-para-pyruvate or with Na-methyl-dihydroxytrimesinate those were prepared by Wolff's method (1899), since kojic acid is suggested to be formed from these subtances if acetaldehyde or pyruvic acid has an important rôle in the formation of kojic acid.

Summary.

(1) The influence of the concentrations of glucose or glycerol and of $(NH_4)_2SO_4$ on the formation of kojic acid by Aspergillus oryzae was observed. More kojic acid was formed with the medium containing a least amount of $(NH_4)_2SO_4$ and a greater amount of glucose or glycerol.

(2) The optimum pH for the formation of kojic acid was found to be about 2.4, and for the growth of the mould was about 5.0.

(3) With 20 days' culture containing 5% of various substanses (in the cases of trioses 0.8% solutions were used), 0.05% $(NH_4)_2SO_4$ and the other mineral matters, the formation of kojic acid was compared.

(4) While the yield of the acid differed due to the nature of the substances, kojic acid was obtained with various carbohydrates, polyhydric alcohols and with gluconic acid. Neither with α–methyl glucoside, rhamnose, fatty acids, ketonic acids, aldehydes, ketones, monohydric alcohols, ketonic alcohols nor with amino acids, the formation of kojic acid was observed.

(5) Among various carbohydrates, a considerable amount (40～10%) of kojic acid was obtained with maltose, cane sugar, glucose, inulin, fructose and with xylose, a less amount (6～1%) with mannose, arabinose and with galactose, while any formation was observed neither with lactose, glyceraldehyde nor with dihydroxyacetone.

(6) Among polyhydric alcohols, formation of kojic acid was observed with sorbitol, dulcitol, glycerol, glycero–β–phosphate and with inositol, but neither with mannitol, erythritol nor with ethylene glycol.

(7) With gluconic acid kojic acid was obtained, but with saccharic acid, glyceric acid or with various monohydroxy acid the formation was not observed.

(8) It is suggested that the effect of Na_2CO_3 or Na_2SO_3, in diminishing the formation of kojic acid from various substances is attributable to the change in pH value of the medium.

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P. S. Challenger, Klein and Walker ascertained in the paper (J. Chem. Soc. (1929), 1498.) which we got just now, that Asp. oryzae produced kojic acid from pentoses (xylose and arabinose).